

CYP3A4*2 Gene Polymorphism and its association with Clinical Phenotyping (temperament) Concept of Unani Medicine Philosophy in Indian Population

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Abstract

Phenotypic heterogeneity is one of the major concerns in personalized medicine. Variations in drug metabolism rates vary in every individual and have been associated with drug metabolizing gene polymorphism. CYP3A4*2 gene polymorphism in drug metabolism has been reported to be associated with a range of diseases. In this regard, the present investigation is aimed to determine the possible association of Unani philosophy of temperaments (clinical phenotypes) with CYP3A4*2 gene polymorphism in patients and controls. In the present study, 800 subjects were analyzed for CYP3A4*2 gene polymorphism by PCR-RFLP technique in four different clinical phenotypes (Sanguine, Phlegmatic, Choleric and Melancholic) as per unani philosophy.

Mutant allelic frequencies of patients and controls of four unani temperaments are sanguine (Damavi) (0.19 vs 0.12), phlegmatic (Balghami) (0.21 vs 0.11), bilious (Safravi) (0.23 vs 0.11) and melancholic (Saudavi) (0.16 vs 0.11) subjects. Safravi ($p < 0.04$) and Balghami ($p < 0.01$) results are statistically significant. Bilious (Safravi) and phlegmatic (Balghami) patients showed significant association with CYP3A4*2 gene polymorphism with 1.76 and 2.04 odds risk. Further such studies are essential in unani regime for providing better treatment for many diseases which require personalized medicine based on phenotype-genotype correlation.

Keywords: Phenotypic heterogeneity, temperaments in Unani philosophy, gene polymorphism, drug metabolism, Unani medicine.

Introduction

Precision medicine is one of the significant goals in the present research era in which a better possible treatment for a particular disease could be provided based on the individual physiology, environmental exposures and genetic behavior. Alternative or traditional systems of medicine are wide spectrum medical systems which provide preventive and rehabilitative healthcare without any side effects of that particular habitat based on the individual physiology.

Nearly 80% of the global population depends on the traditional systems of medicine based on herbs for their day to day healthcare needs.⁴⁹ Re-evaluation of such traditional medicine with philosophy and educational content would yield great assurance for the future benefit of mankind.

Unani medicine is one of the centuries old traditional systems of medical practice in India, benefitting mankind even today. In this system, diseases and its treatment are mainly based on the concept of uniqueness of an individual i.e. physiology of an individual. It has rich treasure of therapeutically active products which have immense potential to treat various diseases as a standalone and adjuvant therapy. Unani philosophy is mainly based upon theory of Humors (Akhlat).¹⁴

According to this holistic system, human body comprises of four humours i.e. (i) *Dam* (blood) as 'hot and moist'; (ii) *Safra* (yellow bile) as 'hot and dry'; (iii) *Balgham* (phlegm) as 'cold and moist' and (iv) *Sauda* (black bile) as 'cold and dry'.⁴⁰

Proper quantity (kamiyat) and quality (kaifiyat) of these four humours are required in proper division for the body to perform its specific functions; any change in these parameters will lead to ill health.³⁸ The mizaj or temperaments of the individuals are given by the words Sanguine (Damvi), Phlegmatic (Balghami), Choleric (Safravi) and Melancholic (Saudavi) on the preponderance of the respective humor.²⁹

The temperaments of the individuals are defined by the changes in physical, physiological and psychological distinctiveness and they are independent of racial, ethnic or geographical considerations. Every individual is different from another with respect to these temperamental differences and treatment is given based on these differences in Unani medicinal system.²⁵ Imbalance in sanguine temperament leads to diseases like reduced intestinal motility, respiratory catarrh, uraemia, gout, diabetes, high cholesterol, asthma, genito-urinary disorders, hypersensitivity and capillary congestion. In the similar manner, alterations in choleric temperament could lead to fevers, infections, rashes, urticaria, hyperacidity, headaches, migraines, eyestrain, stress and cardiovascular disorders.

Phlegm congestion, water retention, oedema, slow digestion, weight gain obesity, poor venous circulation, tendency towards depression could be due to inconsistency in phlegmatic temperament. Relatively disparity in melancholic temperament may cause anorexia, poor appetite, constipation, colon and gas related ailments, wasting, emaciation and dehydration, arthritis, neuromuscular disorders and anxiety.¹⁵ According to

medical depictions of Unani temperaments (clinical phenotypes), the metabolism order was Safravi>Damvi>Saudavi>Balghami.⁵¹

Genetic studies associated with unani regime could provide a better insight for understanding the human body and disease pathogenesis.^{27,28} All these four humors have variable metabolic activities as per Unani philosophy. Thus, we hypothesize genetic connotation with unani medicine which could provide a better means in classifying human population into phenotype clusters. It is also assumed that such studies could provide a better platform for traditional medicine for better understanding of human variations and individual differences in treating various diseases. In this regard, the present study is aimed to evaluate the Unani temperaments with the genetic polymorphism of drug metabolizing enzymes.

Cytochrome P450 (CYP) plays a prominent role in phase I metabolism representing the major pathway for drug oxidation. CYP450 activity may be influenced by several factors including genetic composition of the individual, revelation to some dietary as well as environmental chemicals.⁴⁸ Inter-individual variability in drug response can also be attributed to polymorphism in cytochrome P450 genes. Variations in these genes may even influence the drug metabolism of beta blockers and antidepressants. CYP3A4 is a human cytochrome p450 gene located on the chromosome 7q21.1.⁴ CYP3A4 enzyme is expressed high in human liver. About 60 % of the total hepatic cytochrome p450 activity was also found in some individuals. Other than liver it is also expressed in prostate, breast, gut, colon and small intestine.^{8,24,41} About 50 % of current marketed drugs are metabolized by CYP3A4 enzyme out of more than 50 P450 enzymes.^{8,30}

Earlier studies have reported a tenfold variation in CYP3A4 mediated drug metabolism^{5,39} and 90-fold variability in CYP3A4 protein expression.⁴ CYP3A4 is also involved in the oxidative metabolism of a wide variety of xenobiotics including 45–60 % of clinically used drugs.² CYP3A4 activity can be induced by pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor (PPAR α) and glucocorticoid receptor (GR). Induction could lead to enhanced toxicity when the metabolism of the parent compound is accompanied by increased exposure to a hazardous metabolite¹².

CYP3A4*2 664 T>C (Ser222Pro) SNP in the promoter region appears to be associated with decreased expression and activity (1.7 to 5 fold less)^{13,52} and is related to be risk factor for several cancers^{18,19,42} and oxidative stress related diseases². Though there are several studies relating the CYP3A4*2 (664 T>C) polymorphism with the altered drug metabolism^{44,50} studies related to Unani medicine in association with such genetic variations are not yet done so far. Therefore, the purpose of the present study is to evaluate

the possible association of drug metabolizing enzyme variation (CYP3A4*2 gene polymorphism) with patients and to control with different Unani temperaments in South Indian population.

Material and Methods

A total of 800 subjects were included for the current study. All the subjects were examined by Unani physicians from the OPD clinic of National Research Institute of Unani Medicine for skin diseases (NRIUMSD, Hyderabad) and patients were identified with diabetes, vitiligo, GERD and psoriasis diseases on the basis of clinical history and clinical examination by Unani practitioners.

Subject selection: A total of 800 subjects were selected for the study with 200 subjects (100 patients and 100 controls) of each one clinical phenotype or temperament. 200 sanguine (Damavi) (100 diabetic patients and 100 controls), 200 phlegmatic (Balghami) (100 Vitiligo patients and 100 controls), 200 bilious (Safravi) (100 Gastroesophageal reflux disease (GERD) patients and 100 controls) and 200 melancholic (Saudavi) (100 Psoriasis patients and 100 controls).

Assessment of Temperament: A specially designed case record form (CRF) as per the Unani classical text was designed for assessment of humours and the information obtained was recorded from healthy volunteers as well as patients. Written consent was taken from all the subjects to take part in the research process.

Dominant clinical phenotype or temperament was analysed based on the CRF'S. Healthy individuals of either sex in the age group of 20-55 years who have a positive family history and free from chronic diseases were selected under control group and they were also classified as per four clinical phenotypes. This study was approved by the Institutional Ethics Committee of National Research Institute of Unani Medicine for Skin Disorders(NRIUMSD) (CRIUM formerly), Hyderabad following the principles of Helsenki Declaration for subject consent. After the dominant clinical phenotype assessment, blood sample was collected for further analysis.

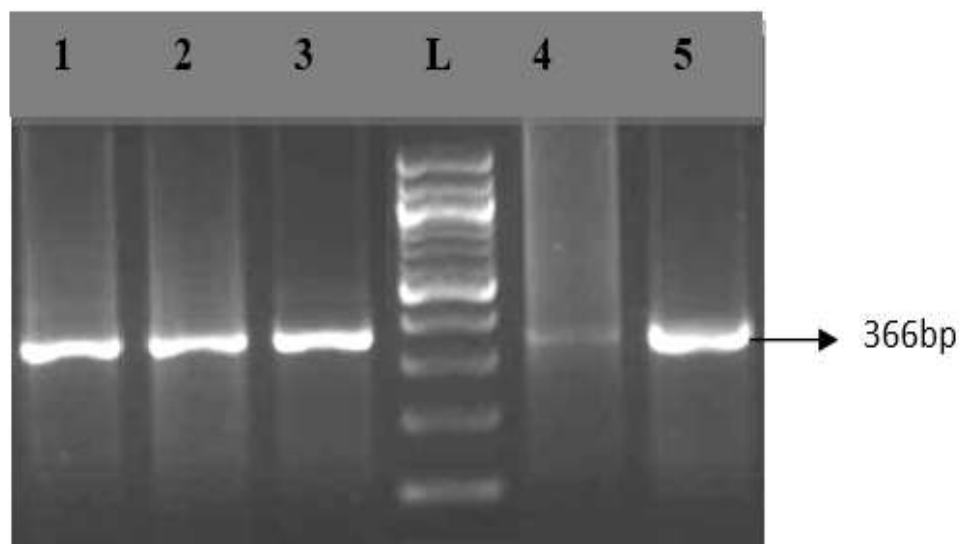
Blood Sample Collection: Two ml of whole blood was collected by veni puncture from all the 800 cases (400 patients and 400 controls) in K2 EDTA vacutainers and transported in an ice box to the molecular biology laboratory. All the samples were stored at -4°C in refrigerator until further use. Written and informed consent was taken from all the subjects before blood sample collection.

DNA isolation: HiPurA™ blood genomic DNA Purification Kit (a column based DNA isolation kit) was used for genomic DNA from peripheral blood samples. The DNA quality and quantity were checked by agarose gel electrophoresis and nanodrop reading by using multimode reader. DNA was then stored at -20°C until further use.

PCR amplification and genotype determination of CYP3A4*2 (664 T>C): The PCR amplification of CYP3A4*2 664 T>C (rs55785340) was performed as illustrated by Suman and Jamil⁴⁴ by using following primer sequence forward: 5'- CCTGTTGCATGCATAGAGG-3' and reverse: 5'GATGATGGTCACACATATC-3'. PCR reaction was performed in a total volume of 30 µl containing 10 mmol/L Tris HCl (PH 8.8), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol of each dNTP, 50 pmol of each primer, 1 unit of Taq DNA polymerase with 200 µg of genomic DNA. Reactions for CYP3A4*2 673 T<C gene were carried out in a thermal cycler (Fermentas Life Sciences, Bangalore, India) consistent with the following program: 95 °C for 2 min, 35

cycles at 95°C for 1 min, 59.5 °C for 1 min, 72 °C for 1 min and 72 for 5 min.

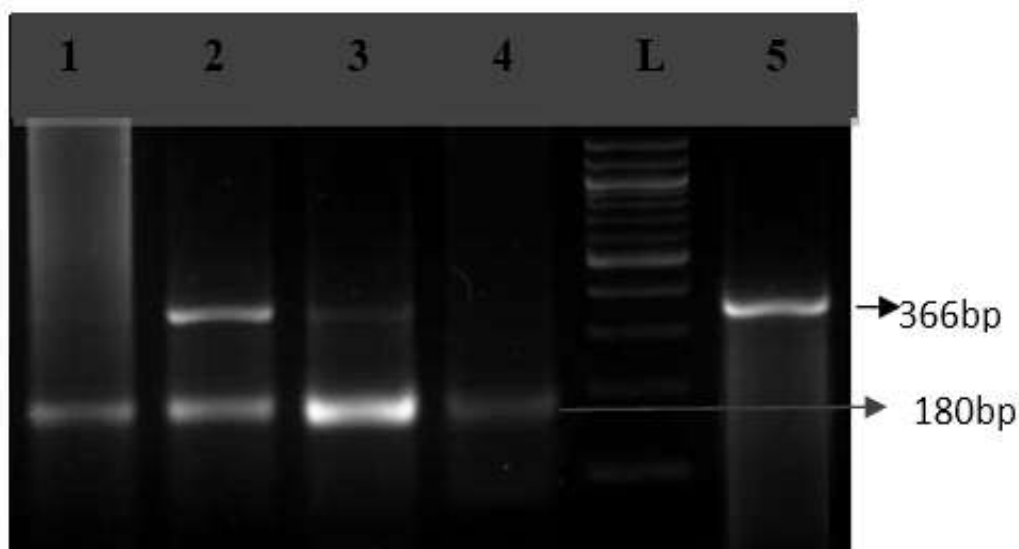
PCR products yielding a fragment of 366 bp were visualized on 2 % agarose gel stained with ethidium bromide before digestion to identify proper amplification of the gene of interest fragments (Figure 1). The CYP3A4*2 genotyping was performed by restriction digestion by HindIII FastDigest restriction enzyme (Thermo Scientific, India). The samples were incubated for 20 min at 37°C and the RFLP products were separated by 3 % agarose gel electrophoresis (Figure 2).



Lane 1,2,3,4 & 5 PCR products 366bp.

Lane L 100bp ladder

Figure 1: CYP3A4*2 (664 T>C) (rs55785340) gene polymorphism PCR products



Lane 1 & 4- TT genotype 180bp

Lane 2 & 3- TC genotype 366bp & 180bp

Lane 5 -CC genotype 366bp

Lane L 100bp ladder

Figure 2: CYP3A4*2 (664 T>C) (rs55785340) gene polymorphism RFLP products

Statistical Analysis: χ^2 test was used to compare the differences in each genotype, allele and combined genotypes frequencies. The risk analysis was performed by calculating Odds Ratio (OR) at 95 % confidence intervals (CIs). A two-tailed p value of <0.05 was considered to be significant. Statistical analysis was performed using openepi software (Version 3.01, April 2013; <http://www.openepi.com>). The observed genotype frequencies for CYP3A4*2 in patients and controls were tested for Hardy–Weinberg equilibrium.

Results

In the present investigation, 800 subjects were enrolled for the study. Temperament wise age and gender distribution were presented in table 1. CYP3A4*1 genotyping was carried out according to PCR-RFLP method for all the subjects.

The genotypic frequencies of CYP3A4*2 664 T>C gene polymorphism of 100 patients and healthy controls of Damvi (Sanguine) temperament were TT 68%, TC 26%, CC 06 % and TT 77%, TC 21% and CC 02% respectively. TT wild genotype showed less frequency in cases compared to controls (68% vs 77 %) respectively whereas the TC genotype and CC genotype were present more in patients compared to controls (26% vs 21 % and 6% vs 2%) respectively (Table 2a). There were no significant differences observed in alleles between the two groups in Damvi (Sanguine) temperament.

CYP3A4*2 664 T>C gene polymorphism genotypic frequencies of 100 patients and healthy controls of Balghami temperament were TT 64%, TC 30%, CC 06 % and TT 79%, TC 19% and CC 02% respectively. TT wild genotype showed less frequency in cases compared to controls (64% vs 79%) respectively whereas the TC and CC genotypes were present more in patients compared to controls (30% vs 19% and 06% vs 02%) respectively (Table 2b). There were significant differences observed in distribution of alleles between the two groups in Balghami temperament with 2.04 odds risk.

CYP3A4*2 664 T>C gene polymorphism genotypic frequencies of 100 patients and healthy controls of Safravi (Bilious) temperament were TT 66%, TC 22%, CC 12 % and TT 75%, TC 21% and CC 04% respectively. TT wild

genotype showed less frequency in cases compared to controls (66% vs 75 %) respectively whereas the CC genotypes were present more in patients compared to controls (12 % vs 04%) respectively (Table 2c). There were significant differences observed in distribution of alleles between the two groups in Safravi temperament with 1.76 odds risk.

The genotypic frequencies of CYP3A4*2 664 T>C gene polymorphism of 100 patients and healthy controls of Saudavi (Melancholic) temperament were TT 72%, TC 23%, CC 05 % and TT 81%, TC 16% and CC 03% respectively. TT wild genotype showed less frequency in cases compared to controls (72% vs 81 %) respectively whereas the TC genotypes were present more in patients compared to controls (23 % vs 16%) respectively (Table 2d). There were no significant differences observed in distribution of alleles between the two groups in Saudavi temperament.

Discussion

Understanding the factors that contribute to the drug action and pharmacodynamic variability of that drug within and between individual human beings for particular diseases, remains a challenge and is still a vision in medical field.²³ Advancements in the pharmacogenomic research along with application of ancient traditional medicine could play a prominent role in the coming future. So far only few studies have been attempted to investigate the relationship between traditional medicine and metabolic gene polymorphisms.^{6,16,37} From past 60 years, several studies on genetic variations in drug metabolizing enzyme genes are being carried out, many of these reported the genetic influence on drug metabolism.^{26,36}

Several previous studies reported the importance of CYP enzymes in drug metabolism.⁵² Since there were no studies related to Unani medicine and genetic variation in drug metabolism, the present study focused on the relationship of drug metabolizing SNP's with four different temperaments in Unani regime. The cytochromes P450 (CYPs) are the major drug metabolizing enzymes which are able to catalyze the oxidative biotransformation of majority drugs and other lipophilic xenobiotics.^{10,31,52}

Table 1
General characteristics

S.N.	Characteristics	Damvi temperament		Balghami temperament		Safravi temperament		Saudavi temperament	
		Controls	Patients	Controls	Patients	Controls	Patients	Controls	Patients
1.	Male	54	55	14	37	48	25	39	67
	Female	46	45	86	63	52	75	61	33
2.	Age (Mean \pm S.D.)	38 \pm 12 years	48 \pm 12 years	37.9 \pm 11.4 years	33.6 \pm 10.8 years	39 \pm 11 years	37 \pm 11 years	39 \pm 11 years	45 \pm 12 years

Table 2a-d

Distribution of allelic and genotype frequencies of CYP3A4*2 (664 T>C) gene polymorphism in patients and control group of different temperament.

Table 2a

Damvi (Sanguine) temperament genotype and allele distribution in Diabetic patients and controls

Genotype/ Alleles	Diabetic patients, n (freq.) (n =100)	Healthy controls, n (freq.) (n = 100)	Odds ratio (95% CI)	p value
TT	68(0.68)	77(0.77)		
TC	26(0.26)	21(0.21)	1.402(0.7238 - 2.715)	0.403
CC	6(0.6)	2(0.2)	3.397 (0.6635 - 17.39)	0.236
Allele				
T	162(0.81)	175(0.875)		
C	38(0.19)	25(0.125)	1.642 (0.9492 - 2.84)	0.09

Table 2b

Balghami (Phlegmatic) temperament genotype and allele distribution in Vitiligo patients and controls

Genotype/ Alleles	Vitiligo patients, n (freq.) (N =100)	Healthy controls, n (freq.) (N = 100)	Odds ratio (95% CI)	p value
TT	64(0.64)	79(0.79)		
TC	30(0.30)	19(0.19)	1.949(1.005-3.78)	0.068
CC	6(0.6)	2(0.2)	3.703 (0.7228 -18.97)	0.192
Allele				
T	158(0.79)	177(0.885)		
C	42(0.21)	23(0.115)	2.046 (1.178 - 3.552)	0.01*

Table 2c

Safravi (Bilious) temperament genotype and allele distribution in GERD patients and controls

Genotype/ Alleles	GERD patients, n (freq.) (n =100)	Healthy controls, n (freq.) (n = 100)	Odds ratio (95% CI)	p value
TT	66(0.66)	75(0.75)		
TC	22(0.22)	21(0.21)	1.19 (0.601 - 2.358)	0.744
CC	12(0.12)	4(0.04)	3.409 (1.049 – 11.08)	0.061
Allele				
T	154(0.77)	171(0.885)		
C	46(0.23)	29(0.115)	1.761 (1.054 – 2.942)	0.0401*

Table 2d

Saudavi (Melancholic) temperament genotype and allele distribution in Psoriasis patients and controls

Genotype/ Alleles	Psoriasis patients, n (freq.) (N =100)	Healthy controls, n (freq.) (N = 100)	Odds ratio (95% CI)	p value
TT	72(0.72)	81(0.81)		
TC	23(0.23)	16(0.16)	1.617 (0.793 - 3.298)	0.251
CC	5(0.5)	3(0.3)	1.875 (0.4328 -8.123)	0.624
Allele				
T	167(0.835)	178(0.89)		
C	33(0.165)	22(0.11)	1.599 (0.8958- 2.853)	0.146

* p value <0.05 is considered as significant

CYP3A4 in P450 enzymes is involved in activation of 60% drugs and many other environmental carcinogens like polycyclic aromatic hydrocarbons, hetero cyclic amines, aflatoxin B1 and nitroso-mines.^{9,35,41} It is also involved in the formation of carcinogen DNA adducts in mammary tissues.²¹ Alternatively, individual variation in CYP3A4 levels can modulate sex hormone metabolite levels and can play a key role in breast and prostate carcinogenesis.

Hirota et al studied the major role of *CYP3A4* in the metabolism of several clinically approved drugs and their results provided an insight for individualized *CYP3A4*-dependent pharmacotherapy. The potential of Pharmacogenomics deals with the identification of genetic variations and role of inheritance in the individual variation in drug response. Genetic factors play an important role in inter individual variability in *CYP3A4* activity.

Felix et al³ reported *CYP3A4* SNPs in drug metabolism as a potential risk factor for carcinomas induced by DNA topoisomerase-II inhibitors. Genotyping of this SNP will be useful in predicting the individuals prone towards altered drug response.

CYP3A4 inhibition by any other exogenous substances like grape fruit could also lead to augmented risk for cardiac diseases. Due to adverse drug reactions arising from grapefruit and terfenadine interaction, terfenadine drug was withdrawn from the market. It was later understood that the inhibition of CYP3A4 enzyme with grapefruit active ingredients led to increased blood terfenadine concentrations leading to prolonged QT intervals following cardiac arrhythmias.^{1,7,11,22,34} Even earlier studies on SNP in the CYP3A4 promoter site revealed significant results for various disease progressions. CYP3A4*2 gene 664 T>C polymorphism and their allelic frequencies between diabetes and control group were found to be significant with disease risk.^{19,43,46}

Conclusion

Present study demonstrates possible genetic relation of CYP3A4*2 gene polymorphism for metabolic differences in the four Unani temperaments (clinical phenotypes) between patients and controls. Identification of such genetic variations underlying drug metabolic variability in patients and controls could provide better approach to Pharmacogenomics. In the current investigation, safravi and Balghami patients showed significant association with CYP3A4*2 gene polymorphism with 1.76 and 2.04 odds risk. Though there are distinguishable differences in genotype frequencies of patients and controls of Saudavi and Damvi, they are not statistically significant.

Further such studies with larger sample size and with other metabolic enzymes should be considered with many gene polymorphisms to provide a better conclusion and help in advancement of traditional medicine particularly Unani System of Medicine (USM) in near future.

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